12-13-99 430 Rec'd PCT/PTO 10 DEC 1999

FORM PTO-1390 REV. 5-93	US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEYS DOCKET NUMBER **P99,2625**
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S.APPLICATION NO. (if known, see 37 CFR 1.5) **09/445796**
INTERNATIONAL APPLICATION NO. **PCT/EP98/04036**	INTERNATIONAL FILING DATE 26 June 1998	PRIORITY DATE CLAIMED 5 July 1997

TITLE OF INVENTION
"ABSORPTION OF MINERALS BY INTESTINAL CELLS"

APPLICANT(S) FOR DO/EO/US
Dominique Brassart and Elisabeth Vey

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay.
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau)
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (unexecuted)

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98; **(PTO 1449, Prior Art, Search Report).**

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.

13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:

EXPRESS MAIL #EL 428 568 115 US, dated December 10, 1999.

420 Rec'd PCT/PTO 10 DEC 1999

U.S.APPLICATION NO. (if known, see 37 C F R 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/445796	PCT/EP98/04036	P99,2625

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):		
Search Report has been prepared by the EPO or JPO $840.00		
International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) . . $670.00		
No international preliminary examination fee paid to USPTO (37 C.F.R. 1.482) but international search fee paid to USPTO (37 C.F.R. 1.445(a)(2) $760.00		
Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2) paid to USPTO $970.00		
International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) . . . . . $ 96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	10 - 20 =	0	X $ 18.00	$	
Independent Claims	3 - 3 =	0	X $ 78.00	$	
Multiple Dependent Claims			$260.00 +	$	
			TOTAL OF ABOVE CALCULATIONS =	$ 840.00	
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 C.F.R. 1.9, 1.27, 1.28)				$	
			SUBTOTAL =	$ 840.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
			TOTAL NATIONAL FEE =	$ 840.00	
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). $40.00 per property +					
			TOTAL FEES ENCLOSED =	$ 840.00	
			Amount to be refunded	$	
			charged	$	

a. ☒ A check in the amount of $ 840.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 08-2290. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Brassart et al. ATTY. DOCKET NO.: P99,2625

SERIAL NO: Unknown

FILED: Herewith

INVENTION: "ABSORPTION OF MINERALS BY INTESTINAL CELLS"

Assistant Commissioner for Patents

Washington, D.C. 20231

CERTIFICATE OF MAILING BY EXPRESS MAIL

Sir:

I hereby certify that the following documents relating to the above-identified application were deposited in the United States Postal Service Express Mail "Post Office to Addressee" on December 10, 1999:

1. Transmittal Letter to the United States Designated/Elected Office (in duplicate);
2. Copy of the Published PCT Application;
3. Declaration and Power of Attorney form (2 pages);
4. Check for $840.00; and
5. Return Receipt Postcard.

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December 10, 1999

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ABSORPTION OF MINERALS BY INTESTINAL CELLS

This invention relates to a method for facilitating or increasing the absorption, by mammals, of minerals from the general diet. In particular, this invention relates to a method which involves the administration of an enteral composition containing *Lactobacilli* micro-organisms.

Minerals are key elements in major physiological processes. Calcium is, for example, of vital importance for the formation of bones and teeth, muscle contraction and the synthesis of hormones. Calcium is also an essential secondary messenger in most cell activation phenomena.

Minerals, of which the diet is the primary source, are assimilated by the body by crossing the intestinal mucosa so as to then pass into the blood stream. The degree of assimilation (or of absorption) of minerals by the body in fact depends both on their solubility in the intestinal medium and on the capacity of the intestinal cells to assimilate them and to transfer them into the blood stream (R. Wasserman *et al., In* Mineral Absorption in the Monogastric GI Trac. Advances in Experimental Medicine and Biology, 249, 45-65, Plenum Press, N.Y., 1989).

The location, the efficiency and the mechanisms of calcium absorption all along the intestine have been studied in rats and chickens for many years (Bronner F., J. Nutr., 122, 641-643, 1992: Schachter D., Am. J. Physiol., 196, 357-362, 1959). For obvious ethical and technical reasons, such studies have been limited in man (Hylander E. *et al.*, Scand. J. Gastroenterol., 25, 705, 1990) and only a few *in vitro* studies have been undertaken (Elsherydah A. *et al.*, Gastroenterology, 109, 876, 1995; Feher J.J., Am. J. Physiol., 244, C303, 1983: Feher J.J., Cell Calcium, 10, 189, 1989).

One of the most widely studied aspects of mineral absorption is the bioavailability of the minerals depending on the composition of the daily diet (Bronner F., J. Nutr., 123, 797, 1993). However, many minerals which are highly bioavailable are also instable and are unsuitable for use in the diet. Further, merely supplementing the diet with greater amounts of minerals often has a negative effect on the organo-leptic properties of the diet.

A possible solution to the problem is to facilitate or improve the absorption of minerals from the diet. However there have been few studies on methods of facilitating or increasing the absorption of minerals from the diet and the results have not been consistent.

Rasic *et al.* have reported that the minerals contained in dairy products are assimilated better when these products are fermented. This effect is attributed to the presence of acids in the fermented dairy products (XP002052238: *In* Fermented Fresh Milk Product, volume 1, p114-115, 1978).

More recently, Yaeshima *et al.* have also shown an increase in the absorption of calcium in rats from a diet of calcium-fortified whey when a combination of oligosaccharides and *Bifidobacteria* is consumed (XP002052237: Bulletin of the International Dairy Fermentation, No. 313, 1996).

However, Kot *et al.* Have reported that *Lactobacillus acidophilus* naturally internalizes Fe^{2+}, and oxidizes it to Fe^{3+}; which is an insoluble form which is more difficult to assimilate (J. Agric. Food Chem., 43, 1276-1282, 1995).

Therefore there remains a need for a means of facilitating or increasing the absorption of minerals present in the diet.

Accordingly, this invention provides a method for increasing absorption of minerals from the diet, the method comprising enterally administering to a mammal a nutritional composition which contains a *lactobacilli* bacteria.

It has been surprisingly found, by use of an *in vitro* model, that *lactobacilli* are able to directly facilitate or improve the absorption of minerals, especially calcium, by human intestinal cells. Without wishing to be bound by theory, this is thought to be linked to induction of acidification of the microenvironment around the intestinal cells and the bacteria in contact with the intestinal cells. Both the bacteria and the intestinal cells may participate in the induction of acidification. This localized acidification might thus play an active role in the solubilization of minerals, and therefore in the capacity of the body to assimilate them.

In another aspect, this invention provides the use of *lactobacilli* in the preparation of an enteral nutritional composition for facilitating or improving the absorption of minerals by the mammal. The enteral nutritional composition may be used for the treatment or prophylaxis of mineral deficiencies

Embodiments of the invention are now described, by way of example only, with reference to the drawings in which:

Figure 1 represents the basal absorption of calcium by Caco-2 intestinal cells in the absence of *lactobacilli*;

Figure 2 represents the influence of about 6.7×10^7 cfu/ml of various strains of *lactobacilli* on the absorption of calcium by Caco-2 intestinal cells;

Figure 3 represents the influence of about $3.4x10^8$ cfu/ml of various strains of *lactobacilli* on the absorption of calcium by Caco-2 intestinal cells.

The invention relates to the enteral administration of a nutritional composition which contains *lactobacilli* to facilitate or improve the absorption of minerals present in a daily diet. Examples of minerals are calcium, magnesium, iron and/or zinc. The ingestion of *lactobacilli* increases the bioavailability of the minerals, that is to say makes the minerals, which are often not very soluble in the intestine, more accessible to the intestinal cells.

Any food-grade, *lactobacilli* strain which may be used. For example, the following *lactobacilli* may be used: *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus amylovorous, Lactobacillus gallinarum, Lactobacillus gasseri* and *Lactobacillus johnsonii; Lactobacillus paracasei; Lactobacillus reuterii; Lactobacillus brevis; Lactobacillus fermentum; Lactobacillus plantarum; Lactobacillus casei* especially *L. casei subsp. casei* and *L. casei subsp. rhamnosus; Lactobacillus delbruckii* especially *L. delbruckii subsp. lactis, L. delbruckii subsp. helveticus* and *L. delbruckii subsp. bulgaricus;* and *Leuconostoc mesenteroides* especially *L. mesenteroides subsp. cremoris*, for example (Bergey's Manual of Systematic Bacteriology, vol. 2, 1986; Fujisawa et al., Int. Syst. Bact. 42, 487-491, 1992).

The *lactobacilli* may be capable of adhering to intestinal cells but need not be. However, the *lactobacilli* are preferably such that at least 50 bacteria, in particular at least 80 bacteria, are capable of adhering *in vitro* to 100 intestinal cells. To select such an adherent type of bacteria, a culture of bacteria may be spread on a confluent culture of an immortalized line of epithelial cells of the intestine (EP 0802257), the confluent culture washed, and the number of bacteria adhering to the villosities of the line measured.

Probiotic *lactobacilli* are of particular interest. Some strains are in fact capable of adhering to human intestinal cells, of excluding pathogenic bacteria which are on human intestinal cells, and/or of acting on the human immune system by allowing it to react more strongly to external aggression (immunomodulation capacity), for example by increasing the phagocytosis capacity of the granulocytes derived from human blood (J. of Dairy Science, 78, 491-197, 1995; immunomodulation capacity of the La-1 strain which was deposited by Nestec SA with the treaty of Budapest in the Collection Nationale de Culture de Microorganisme (CNCM), 25 rue docteur Roux, 75724 Paris,

30 June 1992, where it was attributed the deposit number CNCM I-1225). This strain is described in EP 0577904

By way of example, it is possible to use the probiotic strain *Lactobacillus acidophilus* CNCM I-1225. This strain was recently reclassified among the *Lactobacillus johnsonii* bacteria, subsequent to the new taxonomy, proposed by Fujisawa *et al.*, which is now authoritative in the field of taxonomy of acidophilic lactobacilli (Int. J. Syst. Bact., 42, 487-791, 1992). Other probiotic bacteria are also available, such as those described in EP0199535 (Gorbach *et al.*), US5296221 (Mitsuoka *et al.*), US556785 (Institut Pasteur) or US5591428 (Probi AB), for example.

The nutritional compositions preferably comprise a sufficient quantity of live *lactobacilli* for a facilitated absorption of minerals by the intestinal cells, for example at least 10^6 cfu/ml, in particular 10^7-10^{11} cfu/ml, preferably 10^8-10^{11} cfu/ml ("cfu" means "colony forming unit").

The nutritional composition may also contain other bacteria as desired; for example other probiotic bacteria.

The nutritional composition may also include a suitable protein source; for example an animal or plant protein source. Suitable protein sources are milk proteins, soy proteins, rice proteins, wheat proteins, sorghum proteins, and the like. The proteins may be in intact or hydrolyzed form.

The nutritional composition may also include a suitable carbohydrate source; for example sucrose, fructose, glucose, maltodextrin, and the like.

The nutritional composition may also include a suitable lipid source; for example a suitable animal or plant lipid source. Suitable lipid sources include milk fats, sunflower oil, rapeseed oil, olive oil, safflower oil, and the like.

The nutritional composition may also be fortified with minerals and vitamins. It is especially preferred to fortify the nutritional composition with calcium.

The nutritional compositions may be prepared in the form of food compositions intended for human or animal consumption. Suitable food compositions may be provided in the form of liquids, powders, and solids.

The nutritional composition may be fermented to obtain a sufficient quantity of *lactobacilli*. Fermented compositions based on milk are thus particularly suitable. The term milk applies not only to animal milks but also to what is commonly called a vegetable milk, that is to say an extract of treated or untreated plant materials such as legumes (soya, chick pea, lentil and the like) or

oilseeds (rape, soya, sesame, cotton and the like), which extract contains proteins in solution or in colloidal suspension, which are coagulable by chemical action, by acid fermentation and/or by heat. It has been possible to subject these vegetable milks to heat treatments similar to those for animal milks. It has also been possible to subject them to treatments which are specific to them, such as decolorization, deodorization, and treatments for suppressing undesirable tastes. Finally, the word milk also designates mixtures of animal milks and of plant milks.

It is also possible to add, mix or coat the nutritional composition, during its preparation, with an appropriate quantity of a culture of *lactobacilli* in liquid, concentrated, dry or encapsulated form, according to need.

It has been found that the microencapsulation of the *lactobacilli* has therapeutic advantages. First, microencapsulation significantly increases the survival of the *lactobacilli* and therefore the number of live *lactobacilli* which arrive in the intestine. Even more importantly, the *lactobacilli* are gradually released into the intestine, which permits prolonged action of the *lactobacilli* on the absorption of minerals by the intestinal cells.

Preferably, to encapsulate *lactobacilli*, the *lactobacilli* are freeze-dried or spray-dried (EP0818529), and they are incorporated into a gel consisting, for example, of a solidified fatty acid, a sodium alginate, polymerized hydroxypropylmethylcellulose or polymerized polyvinylpyrrolidone. To this effect, the teaching given in FR2.443.247 is incorporated by reference.

The nutritional compositions need not contain carbohydrates necessary for active fermentation by *lactobacilli* in the intestinal medium. On the contrary, the facilitated absorption of minerals is independent of the fermentative activity of the *lactobacilli*, but rather appears to result from the direct contact between the *lactobacilli* and the intestinal cells. This is thought to induce acidification of the microenvironment and therefore a better solubilization of the minerals.

However, it may be desirable to provide for renewal or specific multiplication of the *lactobacilli* in the intestinal medium so as to prolong the effect of facilitated absorption of the minerals. This may be achieved by adding fibres which facilitate the specific multiplication of *lactobacilli* in the intestinal medium to the nutritional composition. These fibres are soluble and fermentable.

These fibres may be selected from, for example, plant pectins, chito-, fructo-, gentio-, galacto-, isomalto-, manno- or xylo-oligosaccharides or

oligosaccharides from soya, for example (Playne et al., Bulletin of the IDF 313, Group B42, Annual Session of September 95, Vienna).

The preferred pectins are polymers of α-1,4-D-galacturonic acid having a molecular weight of the order of 10 to 400 kDa, which can be purified from carrots or tomatoes, for example (JP60164432). The preferred galacto-oligosaccharides comprise a saccharide portion consisting of 2 to 5 repeating units of structure [-α-D-Glu-(1→4)-β-D-Gal-(1→6)-] (Yakult Honsa Co., Japan). The preferred fructo-oligosaccharides are inulin-oligofructoses extracted from chicory which may comprise, for example, 1-9 repeating units of structure [-β-D-Fru-(1→2)-β-D-Fru-(1→2)-] (WO94/12541; Raffinerie Tirlemontoise S.A., Belgium), or oligosaccharides synthesized from sucrose units which may comprise, for example, a saccharide portion consisting of 2 to 9 repeating units of structure [-α-D-Glu-(1→2)-β-D-Fru-(1→2)-] (Meiji Seika Kasiha Co., Japan). The preferred malto-oligosaccharides comprise a saccharide portion consisting of 2 to 7 repeating units of structure [-α-D-Gal-(1→4)-] (Nihon Shokuhin Kako Co., Japan). The preferred isomaltoses comprise a saccharide portion consisting of 2 to 6 repeating units of structure [-α-D-Glu-(1→6)-] (Showa Sangyo Co., Japan). The preferred gentio-oligosaccharides comprise a saccharide portion consisting of 2 to 5 repeating units of structure [-β-D-Glu-(1→6)-] (Nihon Shokuhin Kako Co., Japan). Finally, the preferred xylo-oligosaccharides comprise a saccharide portion consisting of 2 to 9 repeating units of structure [-β-xyl-(1→4)-] (Suntory Co., Japan), for example.

The quantity of fibres in the nutritional composition depends on their capacity to promote the development of *lactobacilli*. As a general rule, the nutritional composition may contain from 1 to 50% of such fibres (by weight relative to the dry matter). The concentration of *lactobacilli* may be at least 10^3 CFU of *lactobacilli* per g of fibres, preferably 10^4 to 10^7 CFU/g of fibres.

Another advantage provided by the fibres consists in the fact that the intestinal transit is retarded by the fibres. This is particularly the case if the quantity of fibres is large, that is to say of the order of 20-50% relative to the weight of the composition. The *lactobacilli* being gradually eliminated by the action of the intestinal transit, it is possible, in this manner, to prolong the beneficial action of the *lactobacilli* on the absorption of minerals by the intestine.

The nutritional compositions may be in the form of any suitable enterally administered food. For example, the nutritional composition may take the form of a fermented milk (EP0577904), an infant (EP0827697), a fromage frais

(PCT/EP97/06947), a ripened cheese, an ice cream (WO 98/09535), a biscuit filled with a cream (EP704164; EP666031), a dry sausage and/or a pâté (EP689769).

The nutritional compositions may also be in a form suitable for people who cannot tolerate dairy products. These nutritional compositions will not contain allergenic milk derivatives. For example, for children who are allergic to milk proteins, the nutritional composition may be formulated to contain hypoallergenic milk derivatives. These milk derivatives may be in accordance with European directive 96/4/EC which states that in a hypoallergenic milk, the allergenic proteins should be immunologically at least 100 times less detectable than in a nonhydrolysed milk (Off. J. Europ. Comm., NoL49/12, annex point 5.a. 1996; Fritsché *et al.*, Int. Arch. Aller. and Appl. Imm., 93, 289-293, 1990).

The nutritional compositions are particularly suitable for the treatment or prophylaxis of people having mineral deficiencies, or to compensate for physiological deficiencies due to a diet low in minerals, or to satisfy major physiological requirements for minerals in children, pregnant women, women who are breastfeeding and the elderly.

This invention is now further described by means of specific examples. The percentages are given by weight unless otherwise indicated. These examples are given by way of illustration only and do not in any manner constitute a limitation of the invention.

Example 1

- Materials: $^{45}CaCl_2$ is obtained from Amersham, Lucifer yellow from Sigma, collagen I from Centrix Pharmaceuticals, PBS, HEPES and the components of the cell culture medium from Gibco, and the culture supports from Falcon.
- Cell culture: the human cell line Caco-2, isolated from a colon adenocarcinoma, is obtained from American Type Culture Collection (passage 41). The cells are placed in culture in an amount of $4x10^4$ cells/cm^2 in DMEM containing 4.5 g/l of glucose, 20% heat-inactivated foetal calf serum, 1 mg/ml of fungizone, 100 U/ml of penicillin/streptomycin, 200 µg/ml of gentamycin and 1% of nonessential amino acids. The cells are regularly tripsinized and placed in culture again at 1:20. The cells used in the calcium transport experiments are placed in culture at $1x10^5$ cells/cm^2 in permeable inserts previously coated with a layer of collagen I at 50 µg/ml. In all cases, the cells are maintained in a 10% CO_2/90% air incubator at 37°C, and the medium is replaced every two days.

- Viability of the Caco-2 cells: in order to exclude the possibility that the potentiation of the absorption of calcium by the intestinal cells in the presence of *lactobacilli* is due to cellular damage, a portion of each sample serving for the assay of calcium was used for an assay of the hexosaminidase activity (Landegren *et al.*, J. Immunol. Method, 67, 379-378, 1984). This colorimetric test makes it possible to quantify cell lysis and/or death by measuring the hexosaminidase activity released into the supernatant from the cytosol of damaged cells. The results show that in all the experiments, the hexosaminidase activity is equivalent in the presence of *lactobacilli*.

- Permeability of the cellular lawn: the integrity of the lawn formed by the Caco-2 cells at the end of their growth and of their differentiation is evaluated by measuring the transepithelial electrical resistance (TEER) using a voltmeter/ohmmeter Millicell-ERS. The calcium absorption experiments are carried out when this resistance reaches at least 700 ohm x cm^2. The permeability of the cellular lawn during the calcium absorption experiments is evaluated by measuring the level of diffusion (in %) of Lucifer yellow, a molecule which does not cross the cell membrane.

- Transport of calcium: the Caco-2 cells are cultured on inserts for 3 to 5 weeks. On the day of the experiment, the cellular lawn is washed twice in PBS and then the bottom compartment of the insert incorporating the serosa (basolateral pole of the cells) receives 2.5 ml of carrier buffer (140 mM NaCl, 5.8 mM KCl, 0.34 mM NaH_2PO_4, 0.44 mM KH_2PO_4, 0.8 mM $MgSO_4$, 20 mM HEPES, 4 mM glutamine, 25 mM glucose, pH 7.4) supplemented with 2.5 mM $CaCl_2$, whereas the top compartment of the insert incorporating the intestinal lumen (apical pole of the cells) receives 1.5 ml of carrier buffer supplemented with 10 mM $CaCl_2$ and trace amounts of $^{45}CaCl_2$ and Lucifer yellow. The inserts are then placed at 37°C and 50 µl of sample in the bottom and top compartments are removed at regular intervals.

The radioactivity contained in these samples is evaluated by liquid scintillation counting and makes it possible to extrapolate on the quantity of cold $CaCl_2$ absorbed. The basal transport of calcium is expressed as nmol of calcium transported to the bottom compartment of the insert. The diffusion of Lucifer yellow detected by spectrofluorometry in the bottom compartment is expressed in % of the quantity introduced into the top compartment.

- Influence of the *lactobacilli*: the strains *Lactobacillus johnsonii* La1 (CNCM I-1225), La17, La22, La31; *Lactobacillus acidophilus* La10, La18, La31;

Lactobacillus bulgaricus Lfi5, YL8; *Lactobacillus paracasei* ST11; *Lactobacillus gasseri* LGA7; *Lactobacillus reuteri* LR7 and *Streptococcus thermophilus* Sfi20, YS4 (Nestec collection, Lausanne, Switzerland) are placed in culture under anaerobic conditions in MRS broth for *Lactobacillus* or M17 for *Streptococcus* for two times 24 h, washed in PBS and resuspended in carrier buffer before being introduced into the top compartment of the inserts. The Caco-2:bacteria ratio is then about 1:100 according to the tests ($6.7x10^7$ or $3.4x10^8$ cfu/ml in the top compartment of the inserts, for the tests presented in Figures 2 and 3). The absorption of calcium is evaluated according to the protocol mentioned above.

- Results of the basal transport of calcium: a calcium gradient was established in the inserts by introducing 2.5 mM $CaCl_2$ into the bottom compartment, which corresponds to the normal human plasma concentration, and arbitrarily 10 mM $CaCl_2$ into the top compartment, which would correspond to the calcium content of a food diet. As shown by the results of a representative experiment illustrated by Figure 1, the basal absorption of calcium by the Caco-2 cells increases with time to reach up to 600 nmol/insert, comprising about $3x10^6$ cells, after 4 h. As a check for the integrity of the cellular lawn during the experiment, the diffusion of Lucifer yellow was measured and proved to be less than 2%.

- Measurement of the influence of *lactobacilli*: in Figures 2 and 3, the absorption of calcium by the Caco-2 cells is increased significantly in the presence of the adherent *Lactobacillus johnsonii* strains La1 and La22, in the presence of the non-adherent La10 and La18 *Lactobacillus acidophilus* strains, and in the presence of the *L. paracasei* (ST11), *L. gasseri* (LGA7) and *L. reuterii* (LR7) strains.

The capacity of the bacteria to adhere to the intestinal cells therefore does not appear to correlate directly with their capacity to increase the absorption of calcium by these same cells. In all these experiments, the diffusion of Lucifer yellow is modulated in a similar manner but remains negligible.

A decrease in pH in the top compartment of the inserts is also observed when the Caco-2 cells are in the presence of *lactobacilli*, regardless of the strain, except with the Sfi20 strain (Table 1). There is therefore no correlation between the increase in the absorption of calcium and this decrease in pH. However certain bacterial strains capable of increasing the absorption of calcium are not capable of acidifying the experimental medium in the absence of Caco-2. This means that the acidification in the presence of Caco-2 and of bacteria requires a

collaboration between the two types of organisms and could be due to the Caco-2 cells.

Table 1: Influence of *lactobacilli* on the pH of the experimental medium in the absence or in the presence of Caco-2 cells

Bacteria	Number of tests	pH without Caco-2	pH with Caco-2
None	4	7 +/- 0	7 +/- 0
La1	3	6.75 +/- 0.3	3.75 +/- 0.3
La10	3	4.65 +/- 0.3	4.15 +/- 0.3
La17	2	7 +/- 0	3.5 +/- 0.7
La18	2	7 +/- 0	3.5 +/- 0.5
La22	2	7 +/- 0	3.25 +/- 0.35
La29	2	4.25 +/- 0.35	3.5 +/- 0
La31	2	7 +/- 0	3.75 +/- 0.35
Sfi20	1	7	7
YS4	1	5	4
Lfi5	1	4	3
YL8	1	4	3

Example 2

Tests similar to those carried out in Example 1 were carried out to determine the influence of *lactobacilli* on the absorption of calcium by the intestinal cells in the presence of labelled inulin (^{3}H-inulin, Amersham: tracer prebiotic fibre). The results confirm that *lactobacilli* increase *in vitro* the absorption of minerals by the intestinal cells.

Example 3

Tests similar to those carried out in Example 1 were carried out in order to determine the influence of *lactobacilli* on the absorption of magnesium, iron and zinc by the intestinal cells. The results confirm that *lactobacilli* increase *in vitro* the absorption of minerals by the intestinal cells.

Example 4 Encapsulation of lactic acid bacteria

In a 100 l tank, 80 l of culture medium having the following composition, in %, are prepared:

Yeast extract	0.25%
Trypticase	1.00%
Phytone	0.50%
Glucose	1.50%
L-cysteine HCl	0.05%
K_2HPO_4	0.25%
$ZnSO_4$	0.025%
$FeCl_3$	Trace
Water	Balance to 100%

Inoculation is carried out with 1 l of a 20 h culture of *Lactobacillus johnsonii* La1 (CNCM I-1225). The medium is incubated for 12 h at 30°C. The culture broth is centrifuged and 240 g of cells are recovered. They are diluted in 250 ml of skimmed milk supplemented with 7% lactose. The mixture is frozen using liquid nitrogen. The freeze-drying is performed at 40°C overnight. A 5% dispersion of the powder obtained is prepared in hydrogenated vegetable fat having a melting point of 42°C and liquefied at 45°C. The dispersion is injected at 45°C under a pressure of 4 bar, at the same time as liquid nitrogen, in an amount of 1 part of dispersion for 5 parts of nitrogen, at the top of a vertical cylinder 1.5 m in diameter and 10 m high. A container is placed at the bottom of the cylinder, which contains liquid nitrogen in which the microbeads containing the bacteria whose diameter varies between 0.1 and 0.5 m are collected. The microbeads are then placed in a fluidized bed and an alcoholic solution containing 8% zein is sprayed over the bed, in a quantity such that the zein layer formed around the microbeads represents 5% of their weight.

The microbeads are then incorporated into a food composition intended to facilitate the absorption of minerals by the intestinal cells.

Example 5

A concentrated base for ice cream is prepared by mixing at 60-65°C for 20 min about 11% of lactic fat, 8.8% of milk solids (solids-not-fat), 25% sucrose, 5% of glucose syrup and 0.6% of Emulstab® SE30. The base is homogenized at 72-75°C and at 210 bar (2 stages at 210/50 bar), it is pasteurized at 85°C for 22 sec (APV pasteurizer, France, Evreux, 400 l/h), it is cooled to 4°C and 40% of

milk acidified by *Lactobacillus johnsonii La-1* ($5x10^8$ cfu/ml) and *Bifidobacterium longum Bi16* ($3x10^8$ cfu/ml) strains is added thereto. The composition of this concentrated base is presented in the table below.

Ingredients	Composition (kg)	Fat (%)	Solids-not-fat (%)	Sucrose (%)	Dry extract (%)
Cream (35%)	31.43	11.00	1.57		12.57
Skimmed milk powder	7.60		7.30		7.30
Sucrose	36.77			25.00	25.00
Glucose syrup	5.27				5.00
Emulstab® SE30	0.67				0.63
Water	18.26				
Total: cream base	100.00	11.00	8.87	25.00	50.50
Cream base (60%)	60.00	6.60	5.32	15.00	30.30
Acidified milk (40%)	40.00	1.40	4.68	-	6.08
Total: cream base + acidified milk	100.00	8.00	10.00	15.00	36.38

After maturation of the cream for 12 h at 5°C, it is frozen to an overrun of 95% by volume (Crepaco freezer, France, Evreux; 160 l of product/h).

A wafer dough is prepared which contains 10% fructo-oligosaccharide Raftilose® L30 (Raffinerie Tirlemontoise S.A., BE), according to the recipe reproduced in the table below. After baking, the wafer is conventionally formed into a cone. After cooling, the inside of the cones is spray-coated with a fatty film and then the cones are filled with the whipped ice cream described above. For an

11.5 g wafer cone, 130 ml of whipped ice cream (about 65 g) and 5 g of chocolate (spraying over the cream) are thus used.

Ingredient	Weight (g)	Supplier
Ordinary wheat flour 55	52	
Starch	0.2	
Fructo-oligosaccharide Raftilose® L30	10	Raffinerie Tirlemontoise S.A., BE
Sugar	27.8	
Fat	8	
Emulsifier	1.5	
Salt	0.5	
Total: wafer recipe	100	

1.1 g of fibres and about 10^8 cfu/g of *lactobacilli* are thus provided per ice cream cornet. The fibres, by promoting the specific development of *lactobacilli* in the intestinal tract, thus promote the assimilation of minerals.

TRAITE DE BUDAPEST SUR LA RECONNAISSANCE
INTERNATIONALE DU DEPOT DES MICRO-ORGANISMES
AUX FINS DE LA PROCEDURE EN MATIERE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE :

Messieurs ARCHAMBAULT et WAVRE
NESTEC S.A.
Service des Brevets
Avenue Nestlé 55 - CH-1800 VEVEY - SUISSE

RECEPISSE EN CAS DE DEPOT INITIAL, délivré en vertu de la règle 7.1 par l'AUTORITE DE DEPOT INTERNATIONALE identifiée au bas de cette page

NOM ET ADRESSE DU DEPOSANT

NESTEC S.A. - Service des Brevets - Avenue Nestlé 55 - CH-1800 VEVEY - SUISSE

I. IDENTIFICATION DU MICRO-ORGANISME

Référence d'identification donnée par le DEPOSANT :	Numéro d'ordre attribué par l'AUTORITE DE DEPOT INTERNATIONALE :
La 1	I - 1225

II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSEE

Le micro-organisme identifié sous chiffre I était accompagné :

[X] d'une description scientifique

[X] d'une désignation taxonomique proposée

(Cocher ce qui convient)

III. RECEPTION ET ACCEPTATION

La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le 30.06.1992 (date du dépôt initial)[1]

IV. RECEPTION D'UNE REQUETE EN CONVERSION

La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial)
et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion)

V. AUTORITE DE DEPOT INTERNATIONALE

Nom : Collection Nationale de Cultures de Microorganismes Institut Pasteur 25, Rue du Docteur Roux Adresse : 75724 PARIS CEDEX 15	Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : Date : Paris le 02 Juillet 1992 Mme Y. CERISIER Directeur Administratif de la C.N.C.M.

1 En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.

Formule BP/4 (page unique)

TRAITE DE BUDAPEST SUR LA RECONNAISSANCE INTERNATIONALE DU DEPOT DES MICRO-ORGANISMES AUX FINS DE LA PROCEDURE EN MATIERE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE :

Messieurs ARCHAMBAULT et WAVRE
NESTEC S.A.
Service des Brevets
Avenue Nestlé 55 - CH-1800 VEVEY - SUISSE

RECEPISSE EN CAS DE DEPOT INITIAL, délivré en vertu de la règle 7.1 par l'AUTORITE DE DEPOT INTERNATIONALE identifiée au bas de cette page

NOM ET ADRESSE DU DEPOSANT

NESTEC S.A. - Service des Brevets - Avenue Nestlé 55 - CH-1800 VEVEY - SUISSE

I. IDENTIFICATION DU MICRO-ORGANISME	
Référence d'identification donnée par le DEPOSANT : B1 16	Numéro d'ordre attribué par l'AUTORITE DE DEPOT INTERNATIONALE : I - 1228

II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSEE

Le micro-organisme identifié sous chiffre I était accompagné :

[X] d'une description scientifique

[X] d'une désignation taxonomique proposée

(Cocher ce qui convient)

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V. AUTORITE DE DEPOT INTERNATIONALE	
Nom : Collection Nationale de Cultures de Microorganismes Institut Pasteur Adresse : 25, Rue du Docteur Roux 75724 PARIS CEDEX 15	Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : Date : Paris le 02 Juillet 1992 Mme Y. CERISIER Directeur Administratif de la C.N.C.M.

1 En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.

Formule BP/4 (page unique)

Claims

1. Use of *lactobacilli* in the preparation of an enteral nutritional composition for facilitating or improving the absorption of minerals by a mammal.

2. Use according to claim 1 in which the *lactobacilli* is a *Lactobacillus* bacteria which is capable of adhering to intestinal cells.

3. Use according to Claim 2 in which the *lactobacilli* is the *Lactobacillus johnsonii* CNCM I-1225 strain.

4. Use according to Claim 1 in which the enteral nutritional composition contains 10^7 to 10^{11} cfu of *lactobacilli.*

5. Use according to Claim 1 in which the enteral nutritional composition facilitates the absorption of calcium, magnesium, iron and/or zinc.

6. Use according to Claim 1 in which the enteral nutritional composition contains milk proteins.

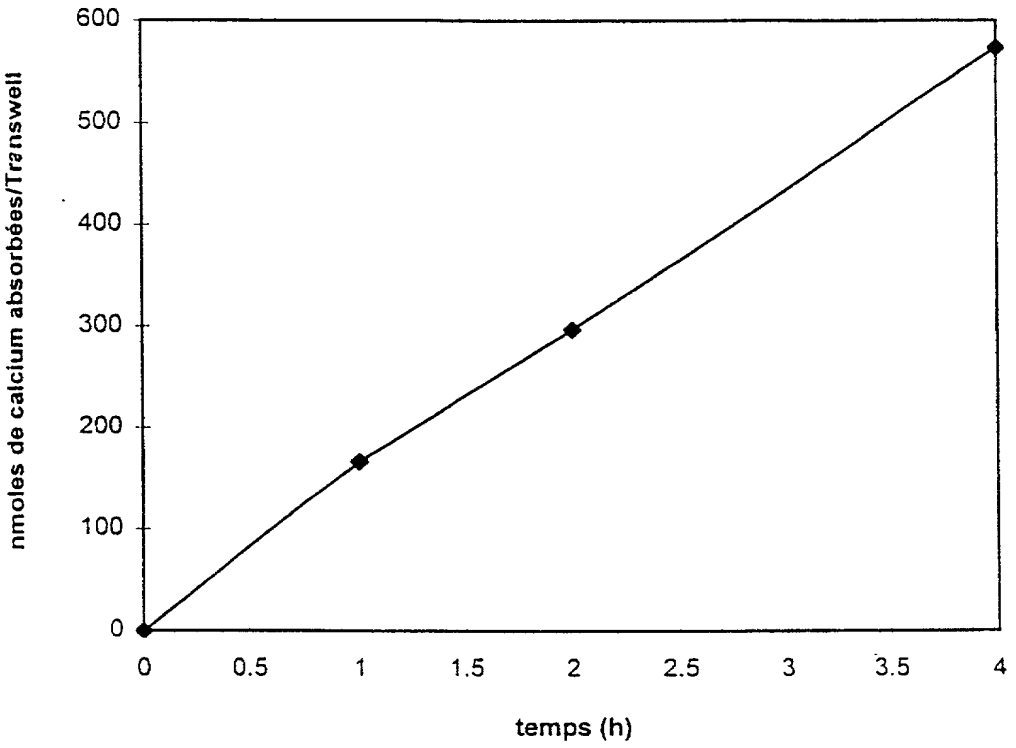
7. Use according to Claim 6 in which the enteral nutritional composition is an infant formula comprising hypo-allergenic milk protein hydrolysates.

8. Use according to Claim 1 in which the enteral nutritional composition further comprises prebiotic fibres.

9. Use of *lactobacilli* in the preparation of an enteral nutritional composition for the treatment or prophylaxis of mineral deficiencies.

10. A method for increasing absorption of minerals from the diet, the method comprising enterally administering to a mammal a nutritional composition which contains *lactobacilli.*

Figure 1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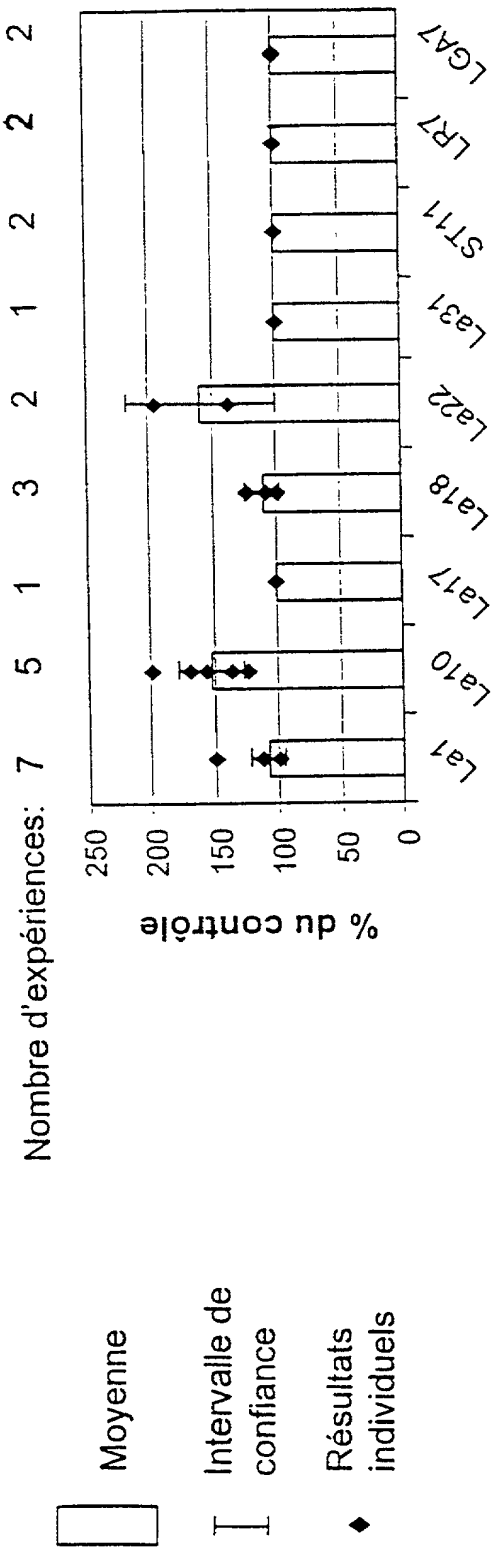
500
400
300
200
100
0
0
0.5
1
1.5
2
2.5
3
3.5
4
temps (h)


Nombre d'expériences: 7 5 1 3 2 2 1 2 2 2
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200
150
100
50
0
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La10
La17
La18
La22
La31
ST11
LR7
LGA7
Moyenne
Intervalle de confiance
Résultats individuels

Figure 2

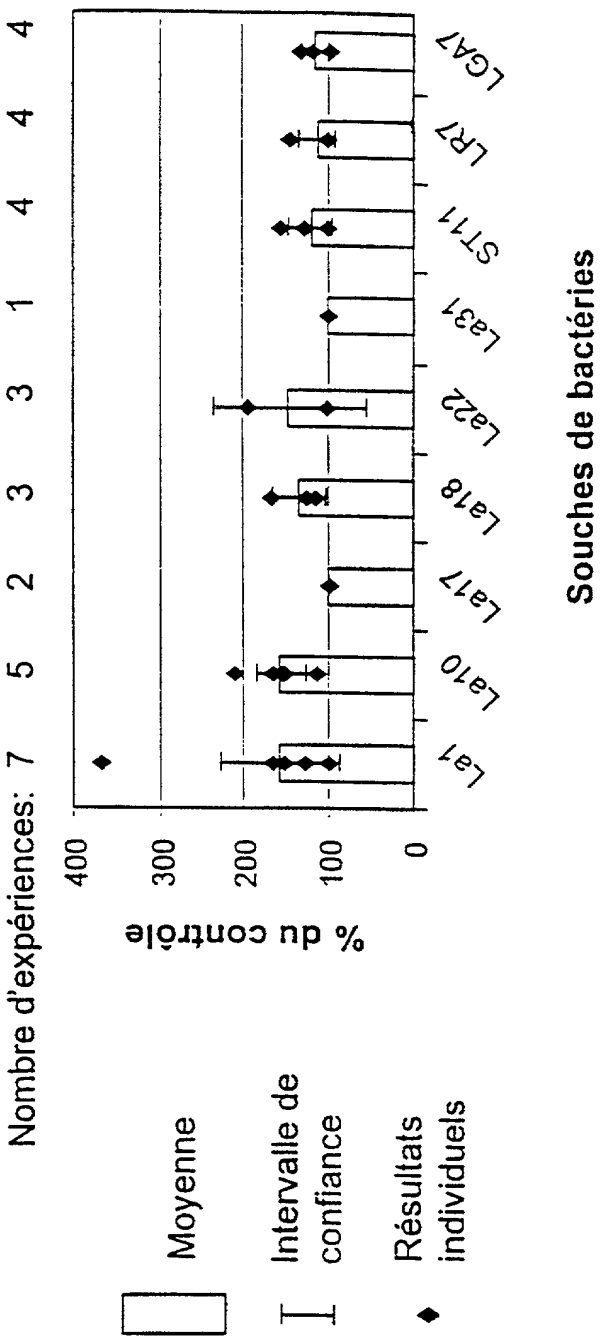

Nombre d'expériences: 7 5 2 3 3 1 4 4 4
400
300
200
100
0
% du contrôle
La1
La10
La17
La18
La22
La31
ST11
LR1
LGA1
Souches de bactéries
Moyenne
Intervalle de confiance
Résultats individuels

Figure 3

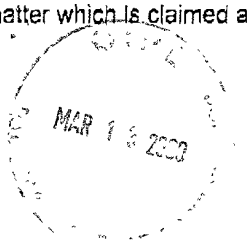
NO 5932/US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER P99,2625

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:



the specification of which (check only one item below):

☐ is attached hereto.

☒ was filed as United States application
Serial No. 09/445,796

on December 10, 1999,

and was amended

on _____(if applicable).

☒ was filed as PCT international application

Number PCT/EP98/04036

on June 26, 1998,

and was amended under PCT Article 19

on _____(if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (If PCT Indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
			☐ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO

Combined Declaration For Patent Application and Power of Attorney (Continued) (Includes Reference to PCT International Applications)	ATTORNEY'S DOCKET NO. P99,2625

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT International application(s) designating the United States of America that is/are listed below and, insofar as the subject mater of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, Untied States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT International filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS		STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)	PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint Jack D. Nimz (Registration No. 35,301) Robert and Gordon N McGrew (37,393), both of Wm. Wrigley Jr Company and Messrs. John D. Simpson (Registration No. 19,842), Lewis T. Steadman (17,074), Dennis A. Gross (24,410), Robert M. Barrett (30,142), Steven H. Noll (28,982), Kevin W. Guynn (29,927), Robert M. Ward (26,517), Brett A. Valiquet (27,841), Edward A. Lehman (22,312), David R. Metzger (32,919), James D. Hobart (24,149), Melvin A. Robinson (31,870), Todd S. Parkhurst (26,494), Joseph P. Reagen (35,332), Michael R. Hull (35,902), Michael S. Leonard (37,557) and Marvin Moody (16,549) all members of the firm of Hill & Simpson, A Professional Corporation

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312/876-0200
Ext. 3060

2 0 0 1	FULL NAME OF INVENTOR	FAMILY NAME: **Brassart**	FIRST GIVEN NAME: **Dominique**	SECOND GIVEN NAME:
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2 0 0 2	FULL NAME OF INVENTOR	FAMILY NAME: **Vey**	FIRST GIVEN NAME: **Elisabeth**	SECOND GIVEN NAME:
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2 0 0 3	FULL NAME OF INVENTOR	FAMILY NAME:	FIRST GIVEN NAME:	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY:	STATE OR FOREIGN COUNTRY:	COUNTRY OF CITIZENSHIP:
	POST OFFICE ADDRESS	POST OFFICE ADDRESS:	CITY:	STATE & ZIP CODE/COUNTRY:

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
DATE 6 mar 2000.	DATE 7. mars 2000	DATE